Regulation of podocalyxin trafficking by Rab small GTPases in 2D and 3D epithelial cell cultures

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「二次元及び三次元上皮細胞培養系における低分子量Gタンパク質Rabを介したポドカリキシンの輸送制御」

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Background

Epithelial tissues line the cavities of blood vessels and organs throughout the body, where they facilitate absorption and secretion, serve as protective barrier, and accommodate sensory receptors. Epithelial cells have a clear apical–basolateral asymmetry characterized by division of their plasma membrane into functionally distinct domains with different composition of proteins and lipids. The apical domain of epithelial cells faces the organ lumen and the basolateral domain is in contact with the underlying extracellular matrix or neighboring cells. The most extensively characterized and the most widely used *in vitro* model of polarized epithelial cells are MDCK (Mardin-Darby canine kidney) II cells (Simmons, 1982). These cells form two-dimensional (2D) monolayers when grown on ordinary culture dishes or develop into three-dimensional (3D) spheroid-like "cysts" with a hollow lumen inside when embedded in extracellular matrix analogues, such as matrigel or collagen.

One of the proteins localized exclusively to the apical domain of MDCK II cells is podocalyxin (PCX). It is also most frequently used as an apical marker in studies on polarization of MDCK cells (Ojakian and Schwimmer, 1988). PCX is a single-pass transmembrane protein with a large, negatively charged, and heavily sialylated and glycosylated extracellular domain, and an evolutionarily conserved cytoplasmic tail with binding sites for several interacting proteins (Nielsen and McNagny, 2008). A single MDCK II cell has a non-polarized distribution of PCX spread all over the plasma

membrane. Upon cell plating on a culture dish or in matrigel, a process of polarity establishment is initiated and PCX is transported from the outer plasma membrane to the newly formed apical domain. As was shown before and confirmed in this study, PCX delivery to the apical domain is required for lumen opening in MDCK II cysts (Bryant et al., 2014; Meder et al., 2005; Mrozowska and Fukuda, 2016). Therefore, PCX is not only a model cargo for studying apical transport but is also crucial for maintaining the morphology of the cyst.

In this thesis, I studied the regulation of the transcytotic pathway of PCX with a special focus on Rab small GTPases.

Section I

Identification of candidate Rab GTPases engaged in podocalyxin trafficking – colocalization screening and knockdown of colocalizing Rabs

Rab GTPases are important coordinators of intracellular trafficking in eukaryotes (Fukuda, 2008; Stenmark, 2009). Thanks to their ability to cycle between an active (GTP-bound) state and an inactive (GDP-bound) state, they function as molecular switches, turning on and off various trafficking steps, e.g. formation, transport, tethering, and fusion of transport vesicles. In this section, in order to identify the candidate Rabs potentially engaged in PCX trafficking in MDCK II cells during polarity establishment, I performed a colocalization screening between PCX and all 60 Rab GTPases. Following the colocalization screening, I knocked down all colocalizing Rabs with specific siRNAs and investigated the PCX localization in Rab-KD cells both under 2D and 3D culture conditions in order to verify which of the Rabs were effectively involved in PCX trafficking. Comparison of KD phenotypes in 2D and 3D culture systems revealed an unexpected finding that, despite the apparently similar trafficking pattern of PCX observed under two culture conditions, PCX trafficking was regulated by different subsets of Rabs.

Section II

Rab35-dependent regulation of PCX trafficking in 2D and 3D culture system

The results presented in Section I revealed that different subsets of Rab GTPases regulated PCX trafficking in 2D and 3D culture systems. Additionally, some of the Rabs that were required both in 2D and 3D cell cultures, when depleted, gave distinct phenotypes. One of such Rabs was Rab35. In this section I further investigated the effect of Rab35 depletion on the morphogenesis of MDCK II epithelial structures using a cell line with completely knocked-out Rab35 by CRISPR/Cas9 system. Rab35-KO MDCK II cysts exhibited the inverted phenotype with PCX being retained at the peripheral plasma membrane, in contrast to the single lumen formation in control MDCK II cells, when grown in the matrigel culture. Such phenotype was not observed at all in cells growing on glass-bottom dishes, in which, even though PCX was trapped in actin-rich clusters *en route* to the apical surface, it was properly internalized. Furthermore, by KD of Rab35 effectors and subsequent KD–rescue experiments for Rab35 mutants, I provided evidence that Rab35 not only gave different phenotypes in 2D and 3D MDCK II cells when depleted, but also acted through different effectors – OCRL in 2D monolayers and ACAP2 in 3D cysts.

Section III

Rab12-dependent exit of PCX from early endosomes

Endocytosed proteins, regardless of the mechanism of their endocytosis, are directed to the major sorting station of endocytic pathways – early endosomes (EE). Sorting events occurring at this compartment determine the fate of endocytosed proteins, destining them for either direct recycling back to the plasma membrane, delivery to the *trans*-Golgi network, or lysosomal degradation. EE are highly dynamic structures, prone to undergo homotypic fusion. They are composed of vacuolar regions with numerous membrane invaginations and regions of tubular extensions, which serve as main sorting hubs. One of the machineries regulating tubulation and cargo sorting at EE is the retromer complex. In this section I showed that PCX was sorted out from EE in a manner dependent on the retromer complex and retromer-associated sorting nexin 27 (Snx27), which bound to the C-terminal tail of PCX. Moreover, this sorting event also depended on the function of Rab12 and its two effector proteins – RILP and RILP-L1. In Rab12-KO MDCK II cells PCX was partially retained in EE and partially mislocalized to lysosomes and to the Golgi apparatus. Depletion of Rab12 also severely abolished formation of FAM21 (another retromer-binding protein)-positive tubular extensions on EE. Based on the findings presented in this section I proposed a following model of Rab12 function in early-endosomal sorting: Rab12, acting through RILP/RILP-L1, links EE membranes to a dynein motor, which provides a pulling force that facilitates early endosomal membrane tubulation required for proper sorting of PCX to recycling endosomes.

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